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International application number: PCT/EP05/003211

International filing date: 21 March 2005 (21.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
                    Number: 60/554,808
                    Filing date: 19 March 2004 (19.03.2004)

Date of receipt at the International Bureau: 02 August 2005 (02.08.2005)
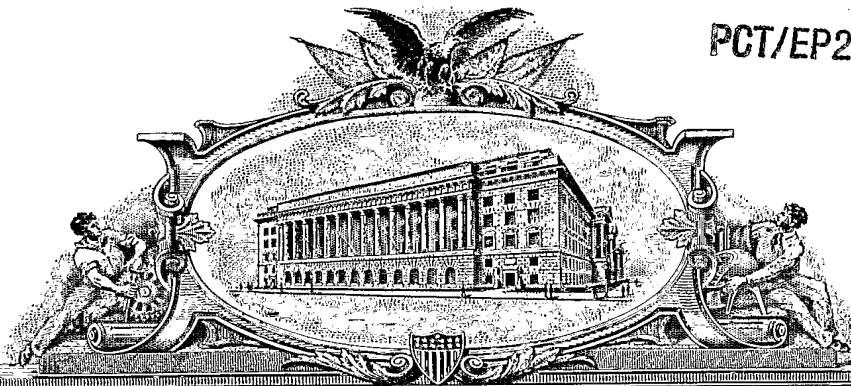

Remark: Priority document submitted or transmitted to the International Bureau in
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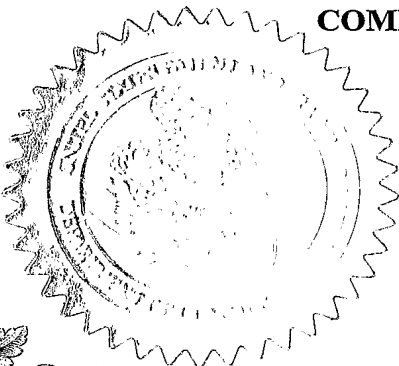**United States Patent and Trademark Office**

May 27, 2005

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APPLICATION NUMBER: *60/554,808*
FILING DATE: *March 19, 2004*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

EXPRESS MAIL NO. EL852794988US

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jörn	**Lewin**	**Berlin, Germany**

☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

A METHOD TO ASSESS MEASUREMENT METHODS QUANTIFYING BASE COMPOSITIONS IN DNA

CORRESPONDENCE ADDRESS

Direct all correspondence to:

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification *Number of Pages* **5**   ☐ CD(s), Number _____

☒ Drawing(s) *Number of Sheets* **5**   ☒ Other (specify) **Fee transmittal sheet in duplicate**

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order for $_____ is enclosed to cover the filing fees.

☒ The Commissioner is hereby authorized to charge filing fees to Deposit Account Number: **04-0258**

☒ The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account Number: **04-0258**

☐ Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____.

Respectfully submitted,			
SIGNATURE	*Bruce A. Kaser*	DATE	**March 19, 2004**
TYPED or PRINTED NAME	**Bruce A. Kaser**	REGISTRATION NO. *(if appropriate)*	**31,531**
TELEPHONE	**206-628-7653**	DOCKET NUMBER:	**47675-**

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL
for FY 2004
Effective 10/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) **80**

Complete if Known	
Application Number	
Filing Date	March 19, 2004
First Named Inventor	Lewin
Examiner Name	
Art Unit	
Attorney Docket No.	47675-

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ None

☒ Deposit Account:

Deposit Account Number: **04-0258**

Deposit Account Name: **Davis Wright Tremaine LLP**

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Fee($)	Small Entity Fee Code	Fee($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80
				SUBTOTAL (1)	($)80

2. EXTRA CLAIM FEES

		Extra Claims		Fee from below		Fee Paid
Total Claims		- 20** =		x	=	
Independent Claims		- 3** =		x	=	
Multiple Dependent					=	

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Fee ($)	Small Fee Code	Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for ex parte reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive – unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Petitions related to provisional applications	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)0

SUBMITTED BY (Complete (if applicable))

Name (Print Type)	Bruce A. Kaser	Registration No. (Attorney/Agent)	31,531	Telephone	206-628-7653
Signature	*[signature]*			Date	March 19, 2004

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This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14.

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A method to assess measurement methods quantifying base compositions in DNA

Jörn Lewin
Epigenomics AG
Berlin

19th March 2004

Method

I hereby explain the method with help of an example experiment used for calibration. In the example experiment the test system was used to assess cytosine/thymine base ratio measurement methods as used in most methylation detection protocols using bisulphite treatment of the DNA.

- First identical regions with local differences are subcloned into plasmids. In the experiment this was an inhomogeneous PCR product from incomplete bisulphite treated DNA that resulted in a mixture of molecules with different C/T proportions at all positions that were cytosine prior to conversion with bisulphite.

- A set of the subclones is sequenced to obtain information about the base composition differences. Other methods to determine these differences can be used but sequencing of the subclones is the most appropriate method. In the experiment we sequenced 96 subclones from one inhomogeneous amplificate.

- A set of subclones is chosen, that compared to each other are different at as many positions as possible relevant for the measurement method to be assessed. For this method a number of two chosen subclones is the minimum but three or more lead to a higher resolution. In the experiment we chose three clones which differed at positions that in the genomic sequence were cytosine and resulted in either cytosine or thymine dependent on the bisulphite conversion (see Fig. 0.1).

- Mixable amounts of the chosen plasmids are gained by cultivation of the subclones and plasmid preparations. The gained plasmid stocks are equilibrated to equal concentrations before mixing.

- The plasmid stocks are mixed in unequal proportions. To gain more test mixtures from the same source the proportions are permuted. Though this is possible with many proportions we suggest to use proportions based on 2^n ; $n \in [0, 1, 2...(cloneNumber - 1)]$. In the experiment we mixed the clones in the proportions 1 : 2 : 4, which resulted in eight equally distributed base compositions from 0/7 to 7/7 in steps of 1/7. Permuting the proportions allowed to generate six different mixtures[1] from the three clones which in this experiment covered many measurement points at different levels (see

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[1] proportion permutations for six different mixtures of three clones: (1:2:4), (2:1:4), (1:4:2), (2:4:1), (4:1:2), (4:2:1)

Fig. 0.2). A choice of four clones might be used for up to 24 mixtures with permutations of the proportions 1 : 2 : 4 : 8 leading to 16 base compositions from 0 to 1 in 1/15 steps.

- The mixtures (in the experiment six) can now be used to assess or calibrate methods which measure the base proportions at specific positions. Results from th emethod to be calibrated or assessed can be compared with expectation values based on known proportions in the test system. An example for this is given in Fig 0.2 b.

Use and advantages

Reproducibility

Once a test system like the one described is established it can easily and cheaply be reproduced with low effort and low risk of changes. More complex systems needing more preparation steps (than concentration measurement and mixing), e.g. random PCR or enzymatic preparation steps, might not be as robust as the provided system and have a high variance from batch to batch. All these characteristics make test systems based on the described method a potential commercial product: easy, reliably and cheap to produce as soon as established.

Different proportions within one mixture

Mixtures of e.g. methylated and unmethylated DNA only provide one defined base proportion to be expected after conversion, it is equal at each position. Any problems that might occur from the fact that other positions have other rates are omitted from such system. The test system described here provides different proportions at different positions within one mixture. Therefore it overcomes the problem of the other system, wherein equal proportions at all positions are used and thereby might bias measurements. In addition this method allows to generate data over a range of measurement points and not only at one defined value, therefore a single mixture can be used to assess the whole range of a measurement method.

Specialized tests based on real DNA patterns

The method allows to generate test systems providing any wanted composition of base proportion at different DNA positions whenever a needed pattern can be found in subclones derived from real samples. This allows to always choose the appropriate subclones for any analysis method the test system will be applied

to. It is e.g. possible to choose stretches that show blocks with equal base proportions at all sites of interest. This way the influence of such blocks (like local co-methylation) on measurement methods can be assessed. The fact that real sample material can be used for the initial step of subclone generation allows to easily reproduce patterns as observed in nature. E.g. for methylation analysis this offers, the opportunity to test sensitive detection methods very precisely and in detail, and allows modeling reality in a more appropriate way than by mixing DNA of 0% and 100% methylation at all positions.

Single method step assessment

The generation of mixtures of e.g. methylated and unmethylated DNA requires several steps until it can be used to assess a measurement method based on e.g. PCR products of bisulphite treated DNA. All these steps influence the real expectation values and the results. 1. the production of methylated DNA may be incomplete and introduce errors. 2. the bisulphite conversion might be incomplete 3. the amplification in the PCR might be biased or have a high variance. All these steps add to any variance and/or bias in the final measurement method to be assessed and cannot easily be separated from it. In contrast the here provided test system allows to asses measurement methods as a whole or its single steps. It therefore provides detailed information about single steps and can locate error sources more easily than methods that provide only an assessment of a whole pipeline of steps.

ABSTRACT

I here present a method that allows to assess and calibrate methods and systems that quantify base compositions at special positions in DNA. The method is characterized by using synthetic, highly reproducible test systems. Said test systems are characterized by a) being built by DNA subclone mixtures, b) providing high numbers of measuring points within one DNA subclone mixture.
The measuring points cover the range of the measurement method to be assessed or calibrated in an evenly distributed manner.

If used to assess a DNA methylation detection method the test system is able to test te outcome of single steps of said method and therefore has a huge advantage compared to methods that can only assess the outcome of multiple steps.

The method is characterized by the use of mixtures of subclones from one and the same DNA region that show base composition differences at positions of interest. The method is further characterized by taking more than two subclones, that among one another are as unequal as possible and mix them in permutations of different portions.
The test system allows to build models with patterns very close to observations in real DNA. An established system can easily be used as a standard for optimization and calibration experiments for different methods and is a potential commercial product.
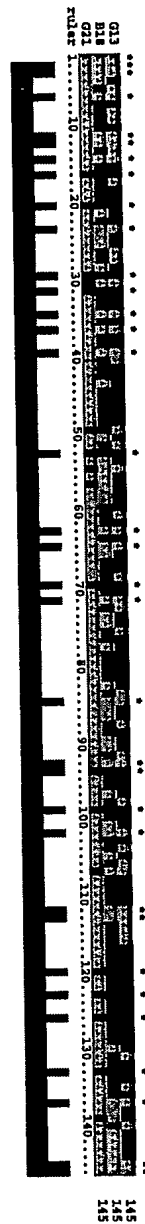
Fig. 0.1: Three final clones chosen for the mixtures, only genomic C positions and their on bisulphite treatment based equivalent (T) are shown.
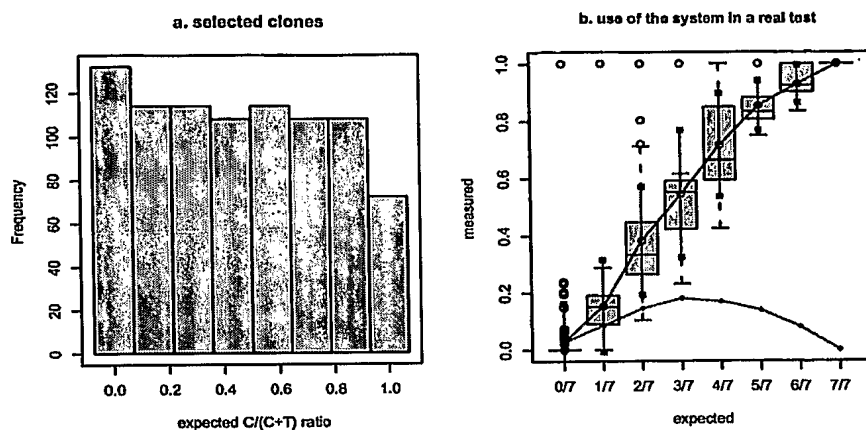
a. selected clones

b. use of the system in a real test

Fig. 0.2: a. Number of measuring points for different C/(C+T) ratios within all six subclone mixtures of the example. b. real calibration data based on an assessment of base ratio detection with four dye capillary sequencing.
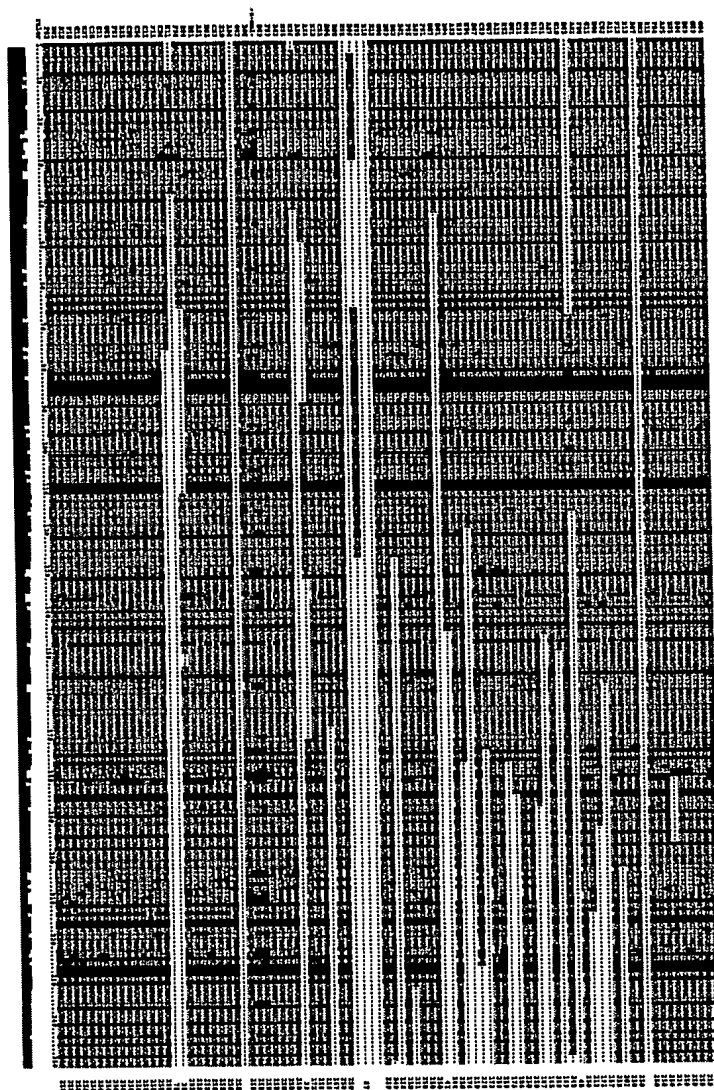
APPENDIX: data from 96 subclones

Fig. 0.3: Full sequence of clones from the initial sub-cloning step of G6e (part 1)
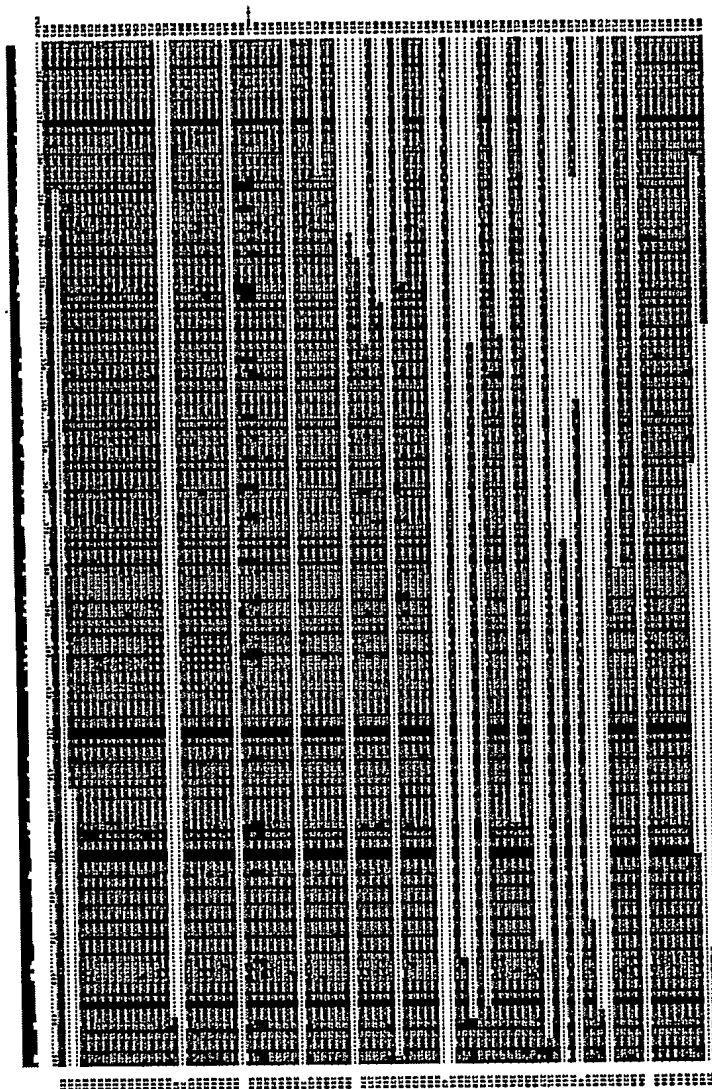
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Fig. 0.4: Full sequence of clones from the initial sub-cloning step of G6e (part 2)
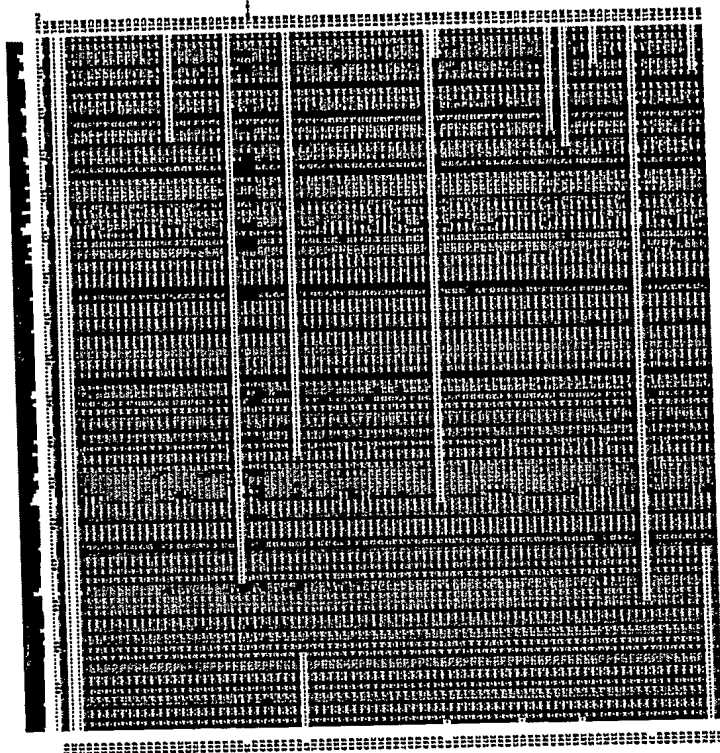
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Fig. 0.5: Full sequence of clones from the initial sub-cloning step of G6e (part 3)